Amendments to the Specification:

Please amend the title of this application, on the cover page, and also on the header of page 1 as indicated.

--~~NUCLEIC ACID SEQUENCES ENCODING TYPE III TENEBRIO ANTIFREEZE PROTEINS AND METHOD FOR ASSAYING ACTIVITY~~ QUANTITATIVE METHOD FOR ASSAYING ANTIFREEZE PROTEIN SPECIFIC RECRYSTALLIZATION INHIBITION ACTIVITY--

Please amend the paragraph beginning on page 2, line 1 as indicated.

--The cryogenic storage of biological samples, cells, tissues and organs, requires cryoprotection for the maintenance of viable cells and cell membranes, that would otherwise be deleteriously affected by the freezing process and storage and recovery from the frozen state. One type of damage that occurs is that cell membranes are susceptible to penetration by ice crystals thereby destroying their function and utility upon ~~warmup~~ warm-up. Such freezing damage may in part be attributed to recrystallization. Likewise, in the area of agriculture, crop yield loss due to frost or freezing can be significant, resulting in the loss of millions of dollars of crops such as oranges and grapefruit. To prevent frost damage, plants may be artificially heated or chemically sprayed resulting in waste of energy or application of chemicals that ordinarily would not have to be applied.--

Please amend the paragraph beginning on page 2, line 19 as indicated.

--The advance of molecular biology techniques have facilitated more biologically based strategies for ice prevention/suppression in a wide variety of applications. For example, U.S. Pat. Nos. 4,045,910 and 4,161,084 to Amy and Lindow disclose protecting plants from frost damage by applying non-ice nucleating bacteria to the plants before the onset of freezing cold. The non-ice nucleating bacteria are supposed to compete with native ice nucleating bacteria and

prevent ice formation by reducing the number of potential "triggers" to crystallization. Following this, Lindow and coworkers (Lindow et al., [1988] Appl. Env. Microbiol. 54:1557-1563) genetically engineered ice minus bacterial mutants for aerial ~~disperal~~ dispersal and competitive exclusion of naturally occurring ice nucleating bacteria for enhanced frost protection. One drawback of this ice prevention method is that it involves the release of genetically modified bacteria into the environment. Alternatively, U.S. Pat. No. 4,834,899 to Klevecz discusses applying a bactericide to plants to prevent frost damage by killing the ice nucleating bacteria while U.S. Pat. No. 4,484,409 to Caple et al. discloses chemically synthesizing polymeric ice nucleation inhibitors via free radical polymerization. The polymers produced in Caple et al. have a tightly controlled spacing of about 15 Angstroms between the hydrophobic and hydrophilic groups. The polymers are sprayed on the plants and are designed to inhibit ice formation. U.S. Pat. No. 4,601,842 to Caple et al. discloses applying naturally occurring biogenic ice nucleation inhibitors, proteinaceous though not characterized further, obtained from cold weather plants to growing crops for protection from frost damage. However, it remains to be seen whether the active agent in these plant extracts is a member of what is now referred to as "antifreeze proteins".--

Please amend the paragraph beginning on page 4, line 36 as indicated.

--Following the discovery of these antifreeze proteins, attempts have been made to exploit the proteins' antifreeze character by using them in biological materials other than those of the fish from which they were derived. As an example, red blood cells were treated with the proteins using standard cryopreservation procedures and exposed to freezing conditions (Carpenter, J. F. and T. N. Hansen [1992] Proc. Nat'l Acad. Sci. 89:8953-8957). The results were highly dependent on concentration of the AFP and in certain concentration ranges actually caused the complete destruction of the cells rather than their preservation, presumably through the spicular ice

growth that occurs in AFP solutions upon reaching the freezing point of the solution. U.S. Pat. No. 5,358,931 to Rubinsky et al., discloses the interaction of AFPs and AFGPs with cell membranes facilitate their cryoprotective effects, while U.S. Pat. No. 5,654,279 to Rubinsky and Koushafar exploit the ~~injurous~~ injurious effects of more elevated titers of AFPs and AFGPs for use in a cryoablation technique of selective tissues damage during cryosurgery.—

Please amend the paragraph beginning on page 5, line 10 as indicated.

--As seen, their utility as efficient ice ~~supressors~~ suppressors, and their proteinaceous nature make THPs an ideal, environmentally friendly material to suppress ice formation in a wide variety of circumstances. Also, they have the advantage that they can be applied, for examples to road surfaces, aircraft wings, or to an agricultural plant ahead of time so that they would interact with ice during formation and, further, they can be applied after the onset of ice formation and serve to prevent continued ice crystal formation. Such upscale uses necessitate a ready source for obtainment of these molecules. In winter flounder, the concentration of AFPs range from 1.0% to 3.0% depending on the species and the season; hence, AFPs are not produced in large enough quantities in arctic fish for the fish to be harvested as a source for an ice prevention agent. There have been some attempts to synthesize AFP using direct chemical processes (Chakrabartty, A. et al. [1989] J. Biol. Chem. 264: 11307-11312); however these processes can be expensive and time consuming.--

Please amend the paragraph beginning on page 8, line 7 as indicated.

--The nucleotide sequences and the predicted amino acid sequences for the peptides are consistent with the earlier amino acid composition assessment (Table 1) showing that the Tenebrio Type II AFP (YL-1), the spruce budworm AFP and the Dendroides AFP all show enriched amino acid residues for

cysteine and threonine, consistent with their being designated as Type II AFPs. Moreover, nucleotide and predicted amino acid sequences of the Type II AFPs from Tenebrio and Dendroides indicate that both sets of proteins are composed predominantly by a series of 12 (Tenebrio) or 13 (Dendroides) amino acid repeats. Additionally, multiple related nucleotide sequences (sharing>80% sequence homology) have been isolated for each of these groups, encoding for numerous isoforms (8 klDa to 20 kDa) from each species. And, these two sets of "repeat sequence" Type II AFPs from Tenebrio and Dendroides were found to share 48-67% identity of residues with one another, corresponding to several conserved regions, which suggest that they are all part of a multigene family encoding these Type II AFPs. In contrast, the sequence analysis of the spruce budworm AFP shows that while being enriched in cysteine and threonine, it bears no similarity to the Type II AFPs from Tenebrio and Dendroides, and also is non-repetitive in sequence. Importantly, however, what the Type II AFPs from all three species do have in common stems from the enriched cysteine and threonine composition of all three. From a comformational perspective, this strongly suggests that these residues are importance in the folded structure and required for the ice binding antifreeze activity. In fact, the disulfide bonded structure is absolutely essential for antifreeze activity in all of these molecules, as disruption of disulfide bridge formation such as treatment with dithiothreitol, results in complete loss of thermal hysteretic activity. The folded structure of the insect Type II Tenebrio and spruce budworm AFPs have recently been reported (Liou, Y. C. et al., [2000] Nature 406:322-324; Graether, S. P. et al., [2000] Nature 406:325-328), as being Beta helical with a triangular cross section and rectangular sides that form stacked ~~parellel~~ parallel Beta sheets. This structural arrangement is quite complex, unlike any seen with the fish antifreeze proteins, and may provide for generating greater thermal hysteretic activities of these insect AFPs over that seen from the fish AFPs and AFGPs. U.S. Pat. Nos. 5,627,051 and 5,633,451 to Duman, regarding Dendroides AFPs and U.S. Pat. No. 6,008,016 to Walker, V. K. et al., regarding the spruce budworm AFPs, disclose nucleic acid and amino acid sequences for their

respective insect Type II AFPs genes and peptides, and application for exploiting the freezing point depression behavior of these antifreeze proteins.--

Please amend the paragraph beginning on page 9, line 14 as indicated.

--Prior to the present invention, unsuccessful attempts had been made at isolating insect Type III AFPs genes. Tang and Baust made use of an antiserum generated against an antifreeze protein active solution derived from *T. molitor*, designated AFP-3 (homogeneity of this peptide was not confirmed) to screen a cDNA *T. molitor* library and isolated a full length clone, the sequence of which was entered into Genbank ~~(NCBI Seq. ID: 785071)~~ (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071). This clone was prematurely (or even incorrectly) listed as an antifreeze protein since recombinant products did not display thermal hysteretic activity. Further support that the AFP-3 clone may not be an antifreeze protein comes from extensive studies by P. Davies and coworkers (Rothemund S. et al., [1997] Biochemistry 36:13791-13801]; [1999] Structure 7:1325-1332), molecular biology experts. In numerous attempts they have cloned the insert generated by Tang and expressed in bacteria the encoded peptide they designated as THP-12 (also known as AFP-3). The recombinant product in all attempts did not display any thermal hysteretic activity, and subsequent NMR spectroscopy studies suggest that the protein has a nonbundle helical structure consisting of six alpha helices arranged in a `baseball glove` shape (i.e. with no obvious ice binding motif seen). They have concluded that THP-12 (AFP-3) might be a member of small lipid carrier class of proteins, yet it[[']]s biological function is as yet undetermined.—

Please amend the paragraph beginning on page 9, line 31 as indicated.

--The present invention successfully isolates insect Type III AFP genes. This was accomplished by using the antiserum generated against Tm 12.86 was to screen newly developed cDNA

libraries prepared from MRNA populations extracted from fat body and whole larvae of winter acclimated T. molitor. Two full length clones (FW-1 and 2-3) were isolated and sequenced. The first clone was found to encode a predicted 18 residue signal peptide proceeding a 116 residue mature peptide of 13.17 kDa molecular weight, that shared 80% amino acid homology with the N-terminal sequence of the endogenous Tm 12.86. Thus, it appeared that rather than isolating the gene encoding Tm 12.86, a homologue (Tm 13.17) was cloned and sequenced. The search of DNA sequence databases revealed that it was most closely related (57% similarity) to the B1 ~~assessory~~ accessory gland protein of T. molitor, and had only moderate (37%) similarity to AFP-3. The recombinant product of the Tm 13.17 clone recovered from the bacterial expression system did not display thermal hysteretic activity. Similarly, a second clone (2-3) was isolated and sequenced and found to encode for a predicted 18 residue signal peptide ~~prececeding~~ preceding a 115 residue mature peptide of 12.84 kDa molecular weight, having a different overall amino acid composition than the native Tm 12.86, but sharing the same N-terminal sequence as Tm 12.86. This clone shares 52% ~~relatenecs~~ relatedness to Tm 13.17 clone, and more moderate (42%) similarity to either the B1 ~~assessory~~ accessory gland protein or AFP-3. Again, the recombinant product did not display thermal hysteretic activity.--

Please amend the paragraph beginning on page 10, line 35 as indicated.

--Another method for assessing AFP efficacy is to monitor the rate of ice crystal growth and morphology microscopically (Raymond, J. et al. [1989], Proc. Nat'l Acad. Sci. USA. 86: 881-885). As noted, one of the most striking and defining physical manifestations of THP presence is the stabilization of seed ice crystals immersed in THP solutions at temperatures maintained within the thermal hysteretic gap. Before stabilization occurs, however, there is evidence to suggest that in certain cases, very limited ice growth does occur initially for crystals. This growth is most evident in THP solutions with low thermal hysteretic activity, and results in

ice crystal morphologies unique to the presence of THPs, hexagonal bipyamids that remain stable as long as temperatures are maintained within the hysteretic gap. However, more potent THP, e.g. the Tm 12.86, Type III insect AFP appears to be capable of stopping ice growth completely before bipyramids form. This, and the non-ease of the assay suggest that crystal morphology analysis of ice growth ~~inhibtion~~ inhibition behavior of THPs in a "non-frozen" solution maintained within the thermal hysteretic gap is not a means for rapid and routine assessment of antifreeze protein activity.—

Please amend the sentence beginning on page 14, line 10 as indicated.

--A general process flow diagram for the present invention can be found in FIG. 1.0.--

Please amend the paragraph beginning on page 15, line 1 as indicated.

--The invention details further the relatedness of this Tm 12.86 AFP multigene family to other known genes, through Genbank searches, establishing that the proteins derived from the Tm 12.84 like clones and Tm 13.17 clone are most closely related (nucleic acid similarity, 43% and 57%, respectively) to B1/B2 accessory gland tubular proteins of adult male *T. molitor*. Also, they are somewhat similar in composition (42% and 37% for Tm 12.84 like and Tm 13.17, respectively) to a lipid carrying protein from Tenebrio designated AFP-3/THP 12 (Tang and Baust, [1995] Genbank NCBI ~~Seq ID: 785071~~ SEQ ID NO:785071; Rothemund et al., [1999]). Despite the latter protein's suggestive abbreviations, the current assessment of it is not that of an antifreeze protein (Rothemund et al., 1999). Finally, the Tm 12.86 AFP family shows no similarity (20%) to the recently isolated Type II AFPs from *T. molitor* and D. canadensis.--

Please amend the sentence beginning on page 16, line 37 as indicated.

--Other uses include, but are not limited to the cosmetic field, and cryosurgery. Additionally, all these effects can be mediated by adding purified THPs alone, or alternatively the THPs can be combined with various "enhancing ~~activitor~~ activator" or adjuvant compounds that are known to enhance THP activity.--

Please amend the paragraph beginning on page 18, line 24 as indicated.

--Commercial uses of the AFPs of the present invention can take on many different facets, some of which are currently being pursued by industry, ~~particulary~~ particularly the frozen food industry and those involved in cryopreservation of cells, tissues, organs, even new tissue engineered biologics, and cryomedicine. The non-colligative freezing point depression activity of AFPs has significant advantage over commercial antifreezes and cryoprotectants including, biodegradability, non-toxicity, and environmental safety. Moreover, these insect Type III AFPs display more potent thermal hysteresis activity than that seen with fish AFPs and AFGP, and are further subject to enhancement by activating substances, also a component of the present invention. The freezing point depression activity of the Tm 12.86 family of peptides, their capabilities of masking potential ice nucleators, ability to stabilize supercooled states, and prevent ice recrystallization, coupled with the ability to clone and express these genes in large amounts of recombinant protein make their applicability and availability for commercial use ideal. Moreover, gene transfer technology for use in generating gene modified organisms (GMO) using AFP genes has broad applicability in agriculture/aquaculture for creating cold-protected, transgenic plants, produce, and fish. On another front, there are numerous applications and advantages to using highly effective, non-toxic antifreeze in de-icing solutions (household, road protection, etc.) and with machinery, e.g., freezer coil de-icing and especially aircraft de-icing. This, coupled with a powerful new means to quantitatively assess ice recrystallization rates and comparative potency evaluations for solutions (from natural or

synthetic sources) conferring antifreeze protein specific inhibition of recrystallization establishes the advantages, benefits and applications of the present invention.--

Please amend the sentence beginning on page 20, line 1 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 1.8 is the N-terminal analysis of Tm 12.86 ~~(SEQ. ID No. 1)~~ (SEQ ID NO:1) depicting leucine at the amino terminus.--

Please amend the sentence beginning on page 21, line 37 as indicated.

--Inserted cDNA can be excised by co-infection with helper phage from the ZAP express vector as a recombinant ~~Bluescript®~~ BLUESCRIPT® SK (-) phagemid (plasmids with a phage origin, sold by Stratagene).--

Please amend the paragraph beginning on page 22, line 3 as indicated.

--FIG. 2.5 is an electrophoresis gel of recombinant pBK-cmv plasmid DNA. The pBK-cmv plasmid DNA containing the cDNA insert was isolated from positive colonies and digested with either one [Lane 2 (4 .mu.g digested by Xho I) and 3 (2 .mu.g DNA digested by Eco R I] or two restriction enzymes [Lane 1 (2 .mu.g DNA digested by Xho I and Eco R I); or no restriction enzyme (Lane 4 (2 .mu.g DNA) and 5 (2 .mu.g DNA). DNA molecule weight standard (3 .mu.g) is shown in Lane 6. The digested DNA was electrophoresed to ~~seperate~~ separate the fragments according to sizes. In Lane 1 two different sizes of fragments, the smaller one (.about.500 bps) is the expected cDNA insert (pointed by arrow) and the larger one was 4518 bp pBK-cmv plasmid vector; Lane 2 shows partially digested DNA byxho I and contained 4 fragments, the largest one was bacterial genomic DNA; the second and the smallest bands represent nicked and supercoiled forms of the ~~recombinat~~ recombinant plasmid respectively; the third one represents

linear form of the recombinant plasmid (<4518 bps) as comparison of bands to non-digested plasmid DNA (lane 4 and 5).--

Please amend the paragraph beginning on page 22, line 16 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 2.6 is a complete sequence of the FW1 clone encoding Tm 13.17 ~~(SEQ.ID NO. 2)~~ (SEQ ID NO:2) and its deduced amino acid (SEQ ID NO:3 and SEQ ID NO:4) ~~(SEQ. ID NOs 3 and 4)~~ of the protein of *T. molitor*. FIG. 2.6A is the full length nucleotide sequence and corresponding deduced amino acid (in single letter nomenclature); The translation start codon, ATG is boxed, and a putative signal peptide sequence are underlined; the stop codon. TGA is in asterisk; polyadenlation signal is in italic and bold, and poly (A) tail is in bold. The arrow indicates the putative cleavage site of the signal peptide. FIG. 2.6B is the signal peptide from deduced amino acid sequence of FW1 cDNA clone. The typical three regions of signal peptide are underlined. The cleavage site is indicated by arrow. FIG. 2.6C is the amino acid sequence and compositional analysis for the predicted mature Tm 13.17.--

Please amend the paragraph beginning on page 22, line 26 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 2.7 illustrates the alignment between the nucleotide cDNA sequences of B1 (Paesen, G. C. and G.M. Happ, [1995] Insect Biochem. Molec. Biol. 25: 401-408) and Tm 13.17 (SEQ ID NO:2) of *T. molitor*. Identical nucleotide sequence is boxed. The start of the mature protein is marked with an arrow, and the stop codons are shown by a star.--

Please amend the paragraph beginning on page 22, line 29 as indicated. Note: Figure designations were underlined in

the original application, and remain unchanged in this amendment.

--FIG. 2.8 illustrates the sequence alignment between mature Tm 13.17 (SEQ ID NO:4) and AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071) of *T. molitor*. Vertical line indicates identical amino acids; two dots indicate highly conservative replacement, and one dot indicates less conservative replacement.--

Please amend the paragraph beginning on page 22, line 32 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 2.9 illustrates the alignment of putative signal peptide sequences of Tm 13.17 (SEQ ID NO:3), AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071) and B1 (Paesen, G. C. and G.M. Happ, [1995] Insect Biochem. Molec. Biol. 25: 401-408) protein of *T. molitor*. The identical amino acid residues and highly conservative replacement are boxed.--

Please amend the sentence beginning on page 22, line 35 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 2.10 illustrates the alignment of N-terminal amino acid sequences of Tm 13.17 (SEQ ID NO:4) and Tm 12.86 (SEQ ID NO:1). The identical amino acids are boxed, dots indicate conservative replacement amino acids.--

Please amend the paragraph beginning on page 23, line 7 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--Fig. 2.12 illustrates the alignment of three amino acid sequences for Tm 13.17 (SEQ ID NO:4), B1 (Paesen, G. C. and G.M. Happ, [1995] Insect Biochem. Molec. Biol. 25: 401-408),

and AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071). 18 N-terminal amino acid residues of Tm 12.86 (SEQ ID NO:1) is also shown in the alignment. The identical amino acid residues are boxed. Note that the arrangement of the proteins from top to bottom (Tm 12.86 (SEQ ID NO:1), Tm 13.17 (SEQ ID NO:4), B1 (Paesen, G. C. and G.M. Happ, [1995] Insect Biochem. Molec. Biol. 25: 401-408), and AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071)) displays first the strong relatedness, and then the falling off identity between the peptides.--

Please amend the paragraph beginning on page 23, line 12 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 3.0 illustrates the cDNA nucleotide sequence ~~(SEQ ID NO 5)~~(SEQ ID NO:5) and amino acid translation of clone 2-2 ~~(SEQ ID NO 7 AND 8)~~(SEQ ID NO:7 and SEQ ID NO:8). The signal sequence is underlined, and the arrow denotes the predicted beginning of the mature protein. The start codon is boxed, and the stop codon is denoted by a star.—

Please amend the paragraph beginning on page 23, line 16 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 3.1 is the cDNA nucleotide sequence ~~(SEQ ID NO 6)~~(SEQ ID NO:6) and amino acid translation of clone 2-3 ~~(SEQ ID NO 7 AND 8)~~(SEQ ID NO:7 and SEQ ID NO:8). The signal sequence is underlined, and the arrow denotes the predicted beginning of the mature protein. The start codon is boxed, and the stop codon is denoted by a star.--

Please amend the paragraph beginning on page 23, line 20 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 3.2[[:]] illustrates comparative nucleotide sequence analysis (SEQ ID NO:5 and SEQ ID NO:6) between clones 2-2& 2-3. Areas of the sequences that are different are boxed.--

Please amend the paragraph beginning on page 23, line 22 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 3.3 illustrates predicted amino acid composition and related information for the peptide derived from clones 2-2/2-3 (SEQ ID NO:8).--

Please amend the paragraph beginning on page 24, line 33 as indicated.

--B. is identical blot to above, but hybridized with the 32P labeled 2-3 probe.--

Please amend the paragraph beginning on page 25, line 11 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.6 illustrates PCR primers used to amplify genomic DNA. FIG. 4.6A illustrates the Tm 13.17 cDNA nucleotide sequence, with the forward and reverse primer sequences boxed (SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4). FIG. 4.6B illustrates representative amino acid sequence alignments of 2-2 (SEQ ID NO:8), Tm 13.17 (SEQ ID NO:4), B2 (Paesen G. C., and G. M. Happ [1995] Insect Biochem. Molec. Biol. 25: 401-408), and AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071). The primer sequences, which only exactly match Tm 13.17, were taken from the boxed areas. FIG. 4.6C illustrates the percent composition and melting temperatures of the forward and reverse primers shown in FIG. 4.6A.--

Please amend the paragraph beginning on page 25, line 27 as indicated. Note: Figure designations were underlined in

the original application, and remain unchanged in this amendment.

--FIG. 4.10A is the cDNA nucleotide sequence ~~(SEQ. ID NO. 9)~~ (SEQ ID NO:9) and translation of 3-4 ~~(SEQ ID NO. 10 (precursor) and SEQ. ID NO. 11 (mature protein)~~ (SEQ ID NO:10) (precursor) and (SEQ ID NO:11) (mature protein). The signal sequence is underlined, and the arrow denotes the predicted beginning of the mature protein. The start codon is boxed, and the stop codon is denoted with a star. FIG. 4.10B is the amino acid composition and related information of the predicted mature 3-4 protein.—

Please amend the paragraph beginning on page 25, line 32 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.11A is the cDNA nucleotide sequence ~~(SEQ. ID NO. 12)~~(SEQ ID NO:12) and translation of 3-9 ~~(SEQ ID NO. 13 (precursor) and SEQ. ID NO. 14 (mature protein)~~ (SEQ ID NO:13) (precursor) and (SEQ ID NO:14) (mature protein). The signal sequence is underlined, and the arrow denotes the predicted beginning of the mature protein. The start codon is boxed, and the stop codon is denoted with a star. FIG. 4.11B is the amino acid composition and related information of the predicted mature 3-9 protein.--

Please amend the paragraph beginning on page 25, line 37 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.12A is the cDNA nucleotide sequence ~~(SEQ. ID NO. 15)~~(SEQ ID NO:15) and translation of 7-5 ~~(SEQ ID NO. 7 (precursor) and SEQ. ID NO. 8 (mature protein)~~ (SEQ ID NO:7) (precursor) and (SEQ ID NO:8) (mature protein). The signal sequence is underlined, and the arrow denotes the predicted beginning of the mature protein. The start codon is boxed, and the stop codon is denoted with a star. FIG. 4.12B is the amino

acid composition and related information of the predicted mature 7-5 protein.--

Please amend the paragraph beginning on page 26, line 3 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.13 illustrates the alignment between the cDNA sequences 2-2, 2-3, 3-4, 3-9, and 7-5 (SEQ ID NO:5; SEQ ID NO:6; SEQ ID NO:9; SEQ ID NO:12; SEQ ID NO:15). Nucleotide residues which disagree are boxed. The start of the mature protein is denoted by an arrow, and the stop codon is marked with a star.--

Please amend the paragraph beginning on page 26, line 6 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.14 illustrates the alignment of the amino acid sequences of 2-2, 2-3, 3-4, 3-9, and 7-5 (SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:13; SEQ ID NO:14), predicted from the nucleotide sequence of the cDNAs. Amino acid residues that differ between sequences are boxed. The arrow denotes the start of the mature protein.--

Please amend the paragraph beginning on page 26, line 11 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.16 illustrates the alignment between the amino acid sequences of Tm 12.86, 2-2, 2-3, 3-4, 3-9, 7-5, Tm 13.17 (SEQ ID NO:1; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:3; SEQ ID NO:4), B1, B2 (Paesen G. C., and G. M. Happ [1995] Insect Biochem. Molec. Biol. 25: 401-408), and AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071). All are sequences obtained from *T. molitor*. All except Tm 12.86 are amino acid sequences

predicted from cDNA nucleotide sequences. The start of the mature protein sequence is at the arrow. Conserved cysteine residues are denoted ~~in yellow~~ by an asterisk. ~~Residues which agree in all nine sequences or ten including the N terminus of Tm 12.86 are in blue.~~ Residues which agree in at least seven proteins are ~~in orange~~ boxed. An open circle denotes a single amino acid deletion in 2-2, 2-3, 3-4, 3-9 and 7-5.--

Please amend the paragraph beginning on page 26, line 18 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 4.17 illustrates the alignment of Tm 13.17 SEQ ID NO:4), 2-2 (SEQ ID NO:8) (representative of 2-2, 2-3, 3-4, 3-9, and 7-5), B1, B2 (Paesen G. C., and G. M. Happ [1995] Insect Biochem. Molec. Biol. 25: 401-408), and eight pheromone binding proteins from various insects (K. Raming, J. Kriegen, and H. Breer [1989] Federation Experimental Biology Letters 256:215-218; T.K. Gyoergyi, A.J. Robi-Shemkovitz and M.R. Lerner [1988] Proc Natn. Acad. Sci, USA 85:9851-9855; R.G. Vogt, R. Rybczynski and M.R. Lerner [1991] J. Neurosci. 11:2972-2984; M.P. McKenna, Hekmal-Scafe, P. Gaines and J.R. Carlson [1994] J. Biol. Chemistry 269:16340-16347; and C.W. Pikielny, G. Hasan, F. Rouyen, and M. Rosbash [1994] Neuron 12:35-49). Arrows ~~above yellow highlighting~~ denotes conserved cysteine residues found in all 12 aligned sequences. ~~Yellow highlighting with no arrow~~ Asterisks denotes cysteine residues conserved in the insect pheromone binding proteins, but not in the B proteins, 2-2, or Tm 13.17. ~~Red shading~~ Other boxed areas show[[s]] agreement between one or more of the Tm 13.17, 2-2, or B1/B2 sequences and any of the representative pheromone or odorant binding proteins. (Pbp: pheromone binding protein; Obp: oderant binding proteins, Antpo (Antherea polyphemus); Manse (M. sexta), Drome, Drosophila melanogaster).--

Please amend the paragraph beginning on page 26, line 27 as indicated. Note: Figure designations were underlined in

the original application, and remain unchanged in this amendment.

--FIG. 4.18 illustrates the areas of repeated similarity surrounding the conserved cysteine residues of 2-2, 2-3, 3-4, 3-9, 7-5, Tm 13.17 (SEQ ID NO:1; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:13, SEQ ID NO:14; SEQ ID NO:3; SEQ ID NO:4), B1, B2, (Paesen G. C., and G. M. Happ [1995] Insect Biochem. Molec. Biol. 25: 401-408) and AFP-3 (Tang and Baust, [1995] GenBank NCBI SEQ ID NO:785071). Conserved cysteine residues are ~~in yellow. Lysine residues are shown in red, glutamate in green, isoleucine in orange, and valine in blue.~~ denoted by an asterisk. Conserved lysine residues (K), glutamate residues (E), isoleucine residues (I), and valine residues (V) are boxed.

Please amend the paragraph beginning on page 26, line 35 as indicated.

--FIG. 4.20 illustrates the phylogenetic tree of the same ~~nucleotde~~ nucleotide sequences displayed in FIG. 4.19.—-

Please amend the paragraph beginning on page 27, line 1 as indicated.

--FIG. 5.2 illustrates the restriction digest screening for pET-2-2 (signal minus insert) in potential clones, demonstrated by the appearance of 350 bp fragment. Also, PBK-CMV double digested to yield a 500 bp fragment served as the positive control. Eighteen potential clones were cultured, ~~mini preped~~ mini-prepped and restriction digested to screen for incorporation of signal-minus fragment. 10 .mu.l of each mini-prep DNA was digested with BAMHI and XhoI and loaded in lanes ~~lableled~~ labeled 1-18 in a 1% agarose gel. Clones in lanes 2, 4, 6, 7, 8, 9, 11, 12, 13 and 18 show a fragment of 350 bp, as marked by the arrow on the right. A positive control pBK-CMV 2-2 was double digested similarly and the 500 bp AFP fragment is seen and denoted by an arrow to the left. The first lane has 1 .mu.g of 100 bp molecular weight marker.--

Please amend the paragraph beginning on page 27, line 24 as indicated.

--FIG. 5.4 illustrates confirmation of signal deleted pET clones with PCR. External primers amplifying 500 bp band in pBK-2-2, 2-3, 13.17, but not in pET; Internal primers amplifying 350 bp band in pET-2-2, 2-3, and 13.17. and pBK. The first two lanes from left are molecular weight markers of 1 kb and 100 bp. Positive controls for the PCR reaction are loaded in Lanes 1, 2 and 3 with pBK-2-2, 2-3 and pET without any insert and Lanes 5, 6, 7 and 8 are 2-2 (S-), 2-3 (S-), Tm13.17 (S+) and Tm13.17 (S-) in pET vector, respectively. The absence of any bands ~~postively~~ positively confirms that there is no contamination from the original vector, pBK-CMV. The second set of samples from Lanes 9 to 16 have been amplified with primers designed to internal sequences of AFP genes. Lanes 9, 10 and 11 are AFP genes in the pBK vector. The amplification of the plasmids confirms the presence of AFP genes. Lane 12 is the pET vector without any insert and the absence of amplified DNA was expected. Lanes 13, 14, 15 and 16 are 2-2 (S-), 2-3 (S-), Tm13.17 (S+) and Tm13.17 (S-) in pET vector, respectively. The presence of a 350 bp fragment confirms the presence of the AFP genes in the pET-28a vector. Lane 15 did not amplify in this gel, but has amplified in other gels (data no shown).--

Please amend the paragraph beginning on page 28, line 1 as indicated.

--FIG. 5.5 illustrates restriction digest screening for pET-2-2 (signal plus) and pET-2-3 (signal plus) in potential clones demonstrated by the appearance of 500 bp fragments. Nine potential clones for 2-2 and nine clones for 2-3 were cultured, ~~min-preped~~ mini-prepped and restriction digested to screen for incorporation of signal plus fragment. 10 .mu.l if each mini-prep DNA was digested with BamHI and XhoI and loaded into lanes ~~labelled~~ labeled 1-18 in a 1% agarose gel. Clones in Lanes 3 and 4 of 2-2 and 11 and 18 of 2-3 release the desired fragment of 500bp Lanes 6 and 8 failed to produce DNA

suggesting that the cultures might be satellite colonies. Molecular weight markers were loaded in the first two lanes with 1 .mu.g of 1kb marker in the first lane and ~~follwed~~ followed by 1 .mu.g of 100 bp marker.--

Please amend the paragraph beginning on page 28, line 10 as indicated.

--FIG. 5.6 illustrates immunoblotting of recombinant proteins of pET: signal plus and signal minus products column purified and thrombin cleaved. Western blot of recombinant products following 15% SDS-PAGE and detection with anti-Tm 12.86 antiserum. A Western blot of recombinant proteins was electrophoresed on a 15% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with milk and incubated first with rabbit anti-Tm12.86 and then incubated with horse radish peroxidase conjugated goat anti-rabbit antibody. Lane 1 ~~depeets~~ depicts molecular weight markers of 46, 29, 20, 14, 8 and 3.5 kD. Lanes 2 and 3 represent 0.1 .mu.l of T. molitor hemolymph and 1 .mu.g of purified Tm12.86, respectively. Lane 4 is 2 .mu.g of whole bacterial lysate from pET 2-2 (S+) and Lanes 5, 6, 7, 8, 9 and 10 represent 1 .mu.g of column purified, thrombin-cleaved, recombinant proteins of pET 2-2 (S+), 2-2 (S-), 2-3 (S+), 2-3 (S-), Tm13.17 (S+) and Tm13.17 (S-), respectively.--

Please amend the paragraph beginning on page 28, line 21 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 5.7 describes the specific cDNA nucleotide sequence ~~(SEQ. ID NO. 16)~~ (SEQ ID NO:16) and translation precursor protein ~~(SEQ ID NO. 17)~~ (SEQ ID NO:17) of His-tagged signal plus 2-2 clone. The signal sequence is underlined, and bold "1" denotes the predicted beginning of the mature protein. The start codon is labeled, and the stop codon is denoted with a star.--

Please amend the paragraph beginning on page 28, line 25 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 5.8 describes the specific cDNA nucleotide sequence ~~(SEQ. ID NO. 18)~~ (SEQ ID NO:18) and translation of mature peptide ~~(SEQ ID NO. 19)~~ (SEQ ID NO:19) of His-tagged signal minus 2-2 clone. The His-tag is upstream of the N-terminal of the mature protein. The bold "1" denotes the predicted beginning of the mature protein. The stop codon is denoted with a star.--

Please amend the paragraph beginning on page 28, line 29 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 5.9 describes the specific cDNA nucleotide sequence ~~(SEQ. ID NO. 20)~~ (SEQ ID NO:20) and translation precursor protein ~~(SEQ ID NO. 21)~~ (SEQ ID NO:21) of His-tagged signal plus 2-3 clone. The signal sequence is underlined, and bold "1" denotes the predicted beginning of the mature protein. The start codon is labeled, and the stop codon is denoted with a star.—

Please amend the paragraph beginning on page 28, line 33 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 5.10 describes the specific cDNA nucleotide sequence ~~(SEQ. ID NO. 22)~~ (SEQ ID NO:22) and translation of mature peptide ~~(SEQ ID NO. 23)~~ (SEQ ID NO:23) of His-tagged signal minus 2-3 clone. The His-tag is upstream of the N-terminal of the mature protein. The bold "1" denotes the predicted beginning of the mature protein. The stop codon is denoted with a star. --

Please amend the paragraph beginning on page 28, line 37 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 5.11 describes the specific cDNA nucleotide sequence ~~(SEQ. ID NO. 24)~~ (SEQ ID NO:24) and translation precursor protein ~~(SEQ ID NO. 25)~~ (SEQ ID NO:25) of His-tagged signal plus Tm 13.17 clone. The signal sequence is underlined, and bold "1" denotes the predicted beginning of the mature protein. The start codon is labeled, and the stop codon is denoted with a star.--

Please amend the paragraph beginning on page 29, line 3 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 5.12 describes the specific cDNA nucleotide sequence ~~(SEQ. ID NO. 26)~~ (SEQ ID NO:26) and translation of mature peptide ~~(SEQ ID NO. 27)~~ (SEQ ID NO:27) of His-tagged signal minus Tm 13.17 clone. The His-tag is upstream of the N-terminal of the mature protein. The bold "1 " denotes the predicted beginning of the mature protein. The stop codon is denoted with a star.--

Please amend the paragraph beginning on page 29, line 19 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

-- ~~FIG. 7-1~~ FIG. 7.1 is a table listing of letter designations for amino acids and chemical classifications.

Please amend the paragraph beginning on page 29, line 21 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--~~FIG. 7-2~~ FIG. 7.2 describes specific details of the nucleotide cons[[c]]ensus sequences developed for the Tm 12.86 family of genes (SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO: 47).--

Please amend the paragraph beginning on page 29, line 23 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--~~FIG. 7-3~~ FIG. 7.3 describes specific details of the protein cons[[c]]ensus sequences encoded by the Tm 12.86 family of genes (SEQ ID NO:48).—

Please amend the paragraph beginning on page 29, line 25 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--~~FIG 8.0~~ FIG. 8.0A illustrates the recrystallization of H_2O after 1 minute; FIG. 8.0B after 30 minutes; and FIG. 8.0C after 2 hours. FIG. 8.0D illustrates the recrystallization of NaCl after 1 minute; FIG. 8.0E after 30 minutes; and FIG. 8.0F after 2 hours. ~~(left) and NaCl (right) occurring after 1 minute, 30 minutes, and 2 hours respectively~~. All samples were annealed at -60.degree. C. (bars=0.1 mm).--

Please amend the paragraph beginning on page 29, line 28 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--~~FIG. 8.1 is a low magnification view of a splat cooled 0.9% NaCl sample annealed at -6.degree. C. for 30 minutes.~~ FIG. 8.1a[[A.]] is a low magnification view of a splat-cooled 0.9% NaCl sample annealed at -6.degree. C. for 30 minutes. Center (c), mid-sample (m), and edge (e) regions are shown. The sample is resting on a support ring (arrow). "th"=thermocouple. (bar at lower right=3.0 mm). FIG. 8.1b[[B.]] is a low magnification view of a splat-cooled 0.9%

NaCl sample annealed at -2.degree. C. for 30 minutes. Putative maximum deformation (mxd) and minimum deformation (mnd) areas are shown. (bar =1.0 mm).--

Please amend the paragraph beginning on page 30, line 3 as indicated.

--FIG. 8.4A is a ~~comparision~~ comparison of mean largest grain size (mlgs) of H.sub.2O (solid) and Tm 12.86, 2.5 ug/ml (~~stipled~~ stippled) taken from different sample regions. FIG. 8.4B are ice grain size heterogeneities for a 0.1 mg/ml BSA in at -6.degree. C. for 2 h and FIG. 8.4B) 0.1 mg/ml alpha lac in H.sub.2O at -2.degree. C. for 2h.--

Please amend the paragraph beginning on page 30, line 7 as indicated.

--FIG. 8.5A illustrates grain size ~~heterogenity~~ heterogeneity of THPs and non-THPs in 0.9% saline. Histogram grouping (left to right). Tenebrio hemolymph (1/1000 dilution), BSA 10mg/ml, BSA 1mg/ml, saline. FIG. 8.5B are low mag (-2.5.times.) of 0.9% NaCl at -6.degree. C. for 30 min (bar -2 mm).--

Please amend the paragraph beginning on page 31, line 19 as indicated.

--FIG. 8.22 is a ~~comparision~~ comparison of RI dilution profiles for Tenebrio hemolymph diluted in saline at -6.degree. C. (square) and -2.degree. C. (diamond). Samples annealed for 30 min.--

Please amend the paragraph beginning on page 32, line 6 as indicated.

--FIG. 8.30 is a comparison of regression lines of RI dilution profiles for winter Tenebrio hemolymph (left line), summer Tenebrio hemolymph (middle line) and T. ~~molior~~ molitor fat body cell culture C1 supernatent (right line). Blank culture media (solid circle) is a control for non-THP RI

effects. Samples diluted in saline and annealed at -6.degree. C. for 30 min.—

Please amend the paragraph beginning on page 32, line 14 as indicated.

--FIG. 8.32 is a ~~comparision~~ comparison of mlgs for R. sylvatica and R. pipens. Samples annealed at -6.degree. C. for 30 min.--

Please amend the paragraph beginning on page 32, line 24 as indicated.

--FIG. 8.36 is a ~~comparision~~ comparison of time course of recrystallization plots for experimental and theoretical prediction.--

Please amend the paragraph beginning on page 32, line 26 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 8.37A is a comparis[[i]]on of time course of recrystallization plots for experimental ~~and theoretical prediction~~ samples using log/log transformations. FIG. 8.37B is a comparision of time course of recrystallization plots for theoretical prediction using log/log transformations. --

Please amend the paragraph beginning on page 33, line 8 as indicated. Note: Figure designations were underlined in the original application, and remain unchanged in this amendment.

--FIG. 8.43 illustrate regions of Tm 13.17 clone used as DNA probes (SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4). ~~Color coded~~ Boxed areas denote forward and reverse ~~prmer~~ primer sequences used for particular experiments with the regions between and including primer sequences denoting the probe. Probe ~~outline by yellow region~~ delineated by symbol 'Y' was used in Example 4, probe delineated by symbol 'G' ~~from green~~

~~region~~ used in Example 5, and probe ~~from pink region~~ delineated by symbol 'P' used for northern analysis.--

Please amend the paragraph beginning on page 33, line 13 as indicated. Note: Figure designations were underlined in the original application, and in this amendment, both Figure designations are now underlined.

--FIG. 8.44 illustrate regions of Tm 2-2 clone used as DNA probes (SEQ ID NO:5; SEQ ID NO:7; SEQ ID NO:8). ~~Color coded~~ Boxed areas denote forward and reverse ~~prmer~~ primer sequences used for particular experiments with the regions between and including primer sequences denoting the probe. Probe usage as in FIG. 8.43.—

Please amend the paragraph beginning on page 33, line 18 as indicated.

--In accordance with the present invention substantially pure peptides (with encoding nucleotide sequences), that exhibit ice crystal growth suppression activity are provided for use in improving or maintaining various characteristics of frozen or chilled foods and biologics, and as environmentally sound de-icing agents. These antifreeze proteins are of an insect Type III AFP classification and are more potent than any of the known fish antifreeze proteins. These insect Type III AFPs can be ~~derivied~~ derived from the natural sources through elimination of contaminating insect compounds or through isolating the desired genes, cloning them, expressing them in a suitable host cell, the purifying the expressed protein, all in a fashion that maintains the peptides non-colligative ice growth suppressing behavior. This invention relates to identifying a multigene family of insect Type III AFPs, providing the isolated nucleic acid sequences encoding this novel class of AFPs, and the generation of these peptides in a manner eliciting antifreeze activity. In addition, the invention also provides for antibodies that are reactive to these peptides, and certain novel activating substances capable of enhancing the antifreeze activity of these Type III insect AFPs are described. Further, the invention details a

sensitive and quantifiable assay for evaluating recrystallization inhibition (RI) that is capable of eliminating non-specific RI effects thereby allowing for an "antifreeze protein specific" response, and means for upscaling the assay. The following illustrate a general detail of and procedures for obtaining the native proteins, the design, preparation, assembly, cloning and expression of deoxyoligonucleotides for use in the manufacture of thermal ~~hysteretically~~ hysterically active recombinant insect Type III antifreeze proteins, the quantitative assay for such, and the use of these antifreeze proteins and related genes and other products.--

Please amend the paragraph beginning on page 36, line 24 as indicated.

--Amino-terminal sequence analysis for Tm 12.86 revealed the sequence for the first nineteen amino acids from the amino terminus ~~SEQ ID NO 1~~ SEQ ID NO:1 and indicated leucine as the amino-terminal amino acid (FIG. 1.8). This result provided added confirmation that Tm 12.86 is a single protein species. To investigate the possibility that a carbohydrate component was associated with Tm 12.86, an additional SDS-PAGE was conducted and stained with PAS.--

Please amend the paragraph beginning on page 40, line 38 as indicated.

--The isolation and characterization of Tm 12.86, and the obtainment of a highly specific and sensitive antibody generated against it, were necessary prerequisites for implementing molecular studies to isolate the gene encoding for this AFP. Steps were taken to construct cDNA libraries from mRNA populations containing the message for Tm 12.86, from whole animal and fat body derived from cold acclimated *T. molitor* larvae according to the procedures detailed in Example 2. Immuno-screening with Tm 12.86 antibody identified a cDNA clone that was subsequently isolated and characterized ~~(SEQ ID NO. 2)~~ (SEQ ID NO:2) and found to encode for a distinct protein, Tm 13.17 ~~(SEQ ID NO. 3~~ (SEQ ID NO:3 (precursor

peptide) ~~SEQ ID NO. 4~~ SEQ ID NO:4 (mature peptide)). The N-terminal sequence of Tm 13.17 shows 61% identity, 83% similarity with that of Tm 12.86 ~~(SEQ ID NO. 1)~~ (SEQ ID NO:1), indicating that this clone is a homologous gene to that of Tm 12.86.--

Please amend the paragraph beginning on page 41, line 22 as indicated.

--Results from spectrophotometer analysis of mRNA indicted the yield of mRNA was about 1 .mu.g out of 100 .mu.g of total RNA, i.e. within the expected range for the amount of mRNA in general. The A260/A280 absorbance ratio of the purified mRNA was 1.8-2.0, thus higher than that from the total RNA. The measure of quality and quantity indicated that the purity was increased after the process of the mRNA isolation. This is supported further with electrophoretic comparison of total RNA, mRNA and RNA remaining after mRNA removal [poly (A.sup.-)] (FIG.2.0). Total RNA before mRNA isolation (lane 1) showed 18S and 28S, which was ~~sharped~~ shaped further in the sample containing RNA minus mRNA (lane 2. In contrast, pure mRNA (lane 3) showed neither 18S or 28S band, but rather a smear bands with several different sizes of mRNA population.--

Please amend the paragraph beginning on page 42, line 3 as indicated.

--To assay for the presence of a labeled Tm 12.86 translated peptide, four samples of the in vitro translation products were subject to immunoprecipitation. FIG. 2.2 presents the Coomassie stained immunoprecipitation samples (lane 1-4) together with ~~orignal~~ original in vitro translation products (lane 6-9) from which the immunoprecipitation products were derived. FIG. 2.3 showed the fluorography of FIG. 2.2 displaying bands (lane 1-2) incorporation .sup.35S-methionine during in vitro translation, and that were immunoreactive to anti-Tm12.86. Also for each figure, samples in lane 1 and 2, and 6 and 7 were derived from T. molitor, while lanes 3 and 4, and 8 and 9 represented control samples,

either containing all components of translation reaction, but without the addition of T. molitor mRNA (to identify any bands not due to the translation products from the mRNA of T. molitor), or another negative control was created by adding dH2O to MRNA of T. molitor instead of the complete in vitro translation reaction mixture. This control checked for contamination of the translation products from the mRNA solution. In FIG. 2.2, immunoprecipitation and in vitro translation products staining with Coomassie showed totally different patterns, yet no visible difference was seen between immunoprecipitation bands from translation products derived from T. molitor mRNA versus those derived from control, establishing consistency in products between samples and the lack of contamination. However, as seen in FIG. 2.3, only one single band (lane 1 and 2) was specifically detected by Tm 12.86 AFP antibody following immunoprecipitation of the T. molitor in vitro translation samples (lane 6 and 7). In contrast, no immunoprecipitation product was detected (lane 3 and 4) when the two control translation samples (lane 8 and 9) were immunoprecipitated with anti-Tm 12.86. Thus a labeled translation product recognized by the antibody to Tm 12.86 was identified as a product of in vitro translation of mRNA isolated from T. molitor. This established mRNA encoding for the Tm 12.86 AFP are present in the mRNA pool of cold acclimated T. molitor. Interestingly, the apparent molecular weight of the immunoprecipitated peptide is about 17 kDa, slightly higher than the purified protein (12.86 kDa) from intact T. molitor or from hemolymph although it must be remembered that translation product represent unprocessed peptides.--

Please amend the paragraph beginning on page 42, line 18 as indicated.

--The 2-2 and 2-3 recombinant proteins are also observed to comigrate with purified Tm 12.86 and Tm 12.86 in hemolymph based on the results of the Western blots. This is a rather unexpected result since the recombinant protein is synthesized as a lacZ-2-2 (or 2-3) fusion protein (the cDNA is inserted within a lacZ gene on the pBK-CMV vector). Since the mature 2-

2/2-3 protein in vivo is putatively 12.84 kD, very similar to the 12.86 kD of the purified THP, it is possible that the amino terminus of the lacZ-2-2/2-3 protein (including signal peptide) was cleaved by an E. coli peptidase. The 2-2/2-3 protein with signal peptide has a molecular weight of about 14.7 kD, which would be expected to migrate at a noticeably slower rate than the 12.86 kD protein. This is not observed on the Western blots, although a set of fainter bands is evident above the 17.8 kD marker indicating the possible presence of some lacZ-2-2/2-3 fusion product (FIG. 3.4). In addition, recombinant Tm 13.17 is also present on the blots as a comparison to 2-2/2-3 and Tm 12.86. The recombinant Tm 13.17 migrates at a ~~discernably~~ discernibly slower rate than 2-2/2-3 or Tm 12.86, with a major band appearing on the Western just below the 17.8 kD marker. Whether or not any post-translational modification of Tm 13.17 in E. coli occurs is difficult to ascertain. Whether or not 2-2 and 2-3 correspond to Tm 12.86 is still uncertain as well. The molecular weight of the putative 2-2/2-3 peptide ("Tm 12.84") is very close to that of Tm 12.86. In addition, amino acid compositions between 2-2/2-3 and Tm 12.86 vary somewhat (Table 3). However, the total number of amino acid residues in the mature 2-2/2-3 peptide is 115, while the total number of residues for Tm 12.86 is 117.--

Please amend the paragraph beginning on page 45, line 38 as indicated.

--DNA sequence analysis and similarity search. All of the seven clones were initially partially sequenced manually from both strands. All were found to have identical DNA sequence. The FW1 clone was then selected for a complete DNA sequence determination of both strands by automatic sequencing. The nucleotide sequence ~~(SEQ. ID No 2)~~ (SEQ ID NO:2) and deduced amino acid sequence ~~(SEQ. ID Nos 3 and 4)~~ (SEQ ID NO:3 and SEQ ID NO:4) of FW1 is presented in FIG. 2.6a. The full length of the cDNA of the FW1 clone is 577 nucleotides long and contains the cloning site E coR I at position 13 and XhoI at position 530. From the partial sequences of the 6 other clones, no sequence heterogeneity was found from that of the clone of

FW1, indicating they all contain the same insert cDNA of *T. molitor*. There is one open reading frame (ORF) from the 577 base pairs. Its start codon ATG is 35 nucleotides downstream from the 5'-end of the clone and the stop codon TGA is at the position of 438 base pair. The 402 nucleotides within encode a peptide containing 134 amino acid residues with a molecular weight of 15.128 kDa. This also includes a putative signal peptide at the N-terminus with 18 amino acid residues, which shows characteristics typical of other signal peptide sequences, including three distinct regions: a basic positively charged N-terminal region (n-region); a central hydrophobic region (h-region) and a more polar C-terminal region (c-region) (FIG. 2.6b). Thus, the predicted mature protein is of 116 amino acid residues ~~(SEQ ID No. 4),~~ (SEQ ID NO:4), with a molecular weight of 13.17 kDa derived from 348 nucleotides. The mature peptide is designated as Tm13.17 for *T. molitor* 13.17 kDa molecular weight. The 3'-end untranslated region of 139 nucleotides is A-T rich (A:T:C:G=55:31:27:26) and presents a AATAAA polyadenylation signal which is located 49 nucleotides downstream of the stop codon and 13 nucleotides upstream of the poly (A) tail. The poly (A) tail occurs 26 nucleotides downstream of the polyadenylation signal.--

Please amend the paragraph beginning on page 47, line 4 as indicated.

--Similarity of the NH2 terminus between Tm 13.17 and Tm 12.86. A comparison of the N-terminal sequence of Tm 13.17 with that determined from protein analysis of Tm 12.86 ~~(SEQ ID NO. 1)~~ (SEQ ID NO:1) indicates a very strong degree of relatedness (FIG. 2.10). 11 out of 18 N-terminal amino acid residues are identical between Tm 13.17 and Tm 12.86. Moreover, in addition to the identical amino acid residues there are 4 highly conservative replacements. Thus, the N-terminus of these two AFPs shows an identity of 61% and similarity of 83%.--

Please amend the paragraph beginning on page 52, line 13 as indicated.

--Nucleotide sequencing for clones 2-2 ~~(SEQ. ID NO. 5)~~ (SEQ ID NO:5) and clone 2-3 ~~(SEQ. ID NO. 6)~~ (SEQ ID NO:6) and predicted amino-acid residues ~~(SEQ. ID NO. 7 and 8)~~ (SEQ ID NO:7 and SEQ ID NO:8) for clones 2-2 and 2-3 are shown in FIG. 3.0 for clone 2-2 and FIG. 3.1 for clone 2-3. The 2-2 cDNA insert consists of a sequence 482 bp. in length, while the 2-3 full cDNA sequence is 483 bp. in length. An evaluation of amino acid translation of the 2-2 cDNA sequence using all six possible reading frames revealed only one likely open reading frame (ORF) consisting of 133 amino acids. An identical amino acid sequence was deduced for 2-3. Toward the start of the ORF for 2-2 and 2-3, a sequence of 18 amino acids corresponds exactly with the amino terminus sequences of Tm 12.86 ~~(SEQ. ID NO. 1,~~ (SEQ ID NO:1, FIG. 1.8). Preceding this 18 amino acid sequence within 2-2 and 2-3 is another 18 amino acids (FIGS. 3.0 and 3.1) that constitute a putative signal peptide characteristic of proteins synthesized for export.--

Please amend the paragraph beginning on page 61, line 2 as indicated.

--A total of five new immunopositive clones were sequenced. Many more positive clones were observed (on average seven per plate in the primary screening), but due to the inherent difficulty in separating the positives from the background plaques, and the need for secondary and tertiary screenings, only five were eventually isolated. Out of the five, two of these clones appear to be false positives, since their sequences are unrelated to Tm 13.17 or 2-2 and 2-3. These may be due to endogenous peroxidases that were not completely knocked out by the peroxide treatment. The remaining three clones were nearly identical in nucleotide sequence to the existing 2-2 and 2-3 clones, and were designated 3-4, 3-9, and 7-5 (FIG. 4.10, 4.11, and 4.12) having ~~SEQ ID NO's 9, 12, and 15~~ SEQ ID NOS:9, 12, and 15 respectively, and encoding for peptides (precursor and mature) having ~~SEQ ID NO's 10-11, 13-14, and 7-8,~~ SEQ ID NOS:10-11, SEQ ID NOS:13-14, and SEQ ID NOS:7-8, respectively for each clone.--

Please amend the paragraph beginning on page 61, line 28 as indicated.

--It is likely that clones nearly identical to one another and to 2-2 and 2-3 make up the largest component of the cDNA library size division 1&2. Positive clones were selected randomly from the cDNA library, yet five out of six of these clones are nearly identical to one another. This suggests that the majority of the clones in this library are very similar to one another. This could be perhaps explained through a repetitive gene duplication event, or 2-2, 2-3, 3-4, 3-9, and 7-5 may be different alleles of the same or similar ~~gens~~ genes, resulting from the polymorphic population used to create the cDNA library.--

Please amend the paragraph beginning on page 67, line 6 as indicated.

--The effect of bacterial proteins on antifreeze activity was evaluated by testing different concentrations of purified antifreeze protein on it's ability to inhibit recrystallization (RI) and the impact (if any) that the presence of bacterial proteins have in this regard (See Example 8 detailing the RI assay). The results were evaluated by visual inspection of photomicrographs. Control sample with no antifreeze protein show large crystals that grew at the expense of smaller sized crystals. When Tm12.86, the positive control was tested in protein extraction buffer its recrystallization ~~inhibiton~~ inhibition activity was preserved at both concentrations i.e. 0.025 mg/mil and 0.0025 mg/ml. Moreover, the average crystal size of 0.025 mg/ml sample was smaller than that of the 0.0025 mg/ml sample. Inhibition of recrystallization was also clearly observed in samples with Tm12.86 in XLOLR lysate. Moreover, the average crystal size with 0.025 mg/ml samples were smaller than that of the more dilute AFP solution. As expected, the negative control with only bacterial lysate did not exhibit any recrystallization ~~inhibtion~~ inhibition as displayed in a pattern similar to that of protein extraction buffer control. The data resulting from these experiments strongly suggested that bacterial proteins

do not hinder the activity of antifreeze proteins. These data do not support the first hypothesis that proposes that bacterial proteins may specifically or non-specifically inhibit antifreeze activity.--

Please amend the paragraph beginning on page 68, line 12 as indicated.

--Generation of Signal Peptide Deleted Fragment(s). Signal peptide deleted fragments were generated by PCR with primers designed to sequences downstream of the signal peptide and upstream of the stop codon. Additionally, two artificial restriction sites, BamHI and XhoI, were designed in the primers in order to incorporate these sites in the fragments ~~(SEQ ID NO's 40-43).~~ (SEQ ID NOS:40-43). The plasmid DNA isolated in the previous step was used as a template in the PCR reaction. Following PCR, the entire reaction product was then electrophoresed on a 1.5% agarose gel, and a distinct and strong band was observed at 350 bp. Since this is the expected size of the AFP clones when the signal peptide, poly-A tail and other non-coding regions are removed, this result suggests that the primers and the PCR reaction successfully yielded a signal deleted cDNA fragment.--

Please amend the paragraph beginning on page 70, line 26 as indicated.

--Sequencing of PET-AFP vectors. For final confirmation that signal-plus and signal-deleted inserts were successfully subcloned into the pET vector without accruing mutations, the sequence analyses of plasmids were performed. Plasmids from bacterial stocks of pET-AFP clones were extracted using procedures detailed in Example 5. The plasmids were amplified by using the T7 promoter sequence found in the upstream region of the multiple cloning site. Following this, sequence analysis of the clones was conducted on a ABI Prism Sequencer. The positive control was pET vector without any insert. The results were compared with the original sequences and were found to have no error. Some sequences were unrecognized by the software and manually read and verified for accuracy. In

addition, the sequences encoding for the histidine tag, the thrombin cleavage site and the 17 tag were preserved in all the clones. The sequencing results of pET-[2-2S+, 2-2S-, 2-3S+, 2-3S-, Tm 13.17S+ and Tm 13.17S-] are presented in FIGS. 5.7-5.12 ~~(SEQ ID NO's 16-27)~~ (SEQ ID NOS:16-27) FOR NUCLEOTIDE AND PEPTIDE SEQS.--

Please amend the paragraph beginning on page 75, line 24 as indicated.

--Importance of Inclusion Body Isolation. When the denaturing and refolding procedures followed in Example 6 are employ on recombinant proteins obtained from the ~~supematent~~ supernatant (as in Example 5), the recombinant proteins still fail to display antifreeze activity. Thus, something associated with the packaging into, and/or the microenvironment of, the inclusion bodies is essential for establishing antifreeze activity of the Tm 12.86 family of Type III AFPs.—

Please amend the heading beginning on page 77, line 34 as indicated.

--VII. ~~Concensus~~ Consensus Sequences for the Tm 12.86 Gene and Protein Family--

Please amend the paragraph beginning on page 77, line 35 as indicated.

--Cons[[c]]ensus sequences for the genes and proteins of the Tm 12.86 family (cladistic tree shown in FIG. 4.20) were identified as detailed in Example 7 paying careful attention to the types of substitutions and chemistry involved. Both a full general cons[[c]]ensus sequence was described for the entire Tm 12.86 gene family encoded proteins, and consensus sequences for the nested genes within the family are also described (i.e. cons[[c]]ensus sequence for Tm 12.84-6 like, consensus sequence expanded to include Tm 13.17 like, cons[[c]]ensus sequence expanded to include B1/B2 like, and cons[[c]]ensus sequence expanded to include AFP-3 like, genes

and their encoded proteins ~~(SEQ ID NO's 44-48).~~ (SEQ ID NOS:44-48). Detailed in FIGS. 7.2 and 7.3 are the full breath of the cons[[c]]ensus sequences for nucleotides and amino acids, respectively, and for each grouping the most representative cons[[c]]ensus sequence, and also positions and types of substitutions either occurring or deemed acceptable. See FIG. 7.1 for reference to amino acid letter designations and chemical classifications.--

Please amend the paragraph beginning on page 78, line 9 as indicated.

--The 5 clones in this series are highly conserved. At the protein level, one (3-9) shows a substitution at position 37 (from the initial methionine) of an amino acid with an acidic side chain (glutamic acid) for one with an aliphatic side chain (valine). Since valine is the most common, it is placed in the cons[[c]]ensus sequence, with the understanding that glutamic acid is a recognized substitution for this gene family. Clone 3-9 also shows a substitution at position 69 of an amino acid with a basic side chain (arginine) for another with a basic side chain (lysine). Again, since lysine is most common, it is included in the cons[[c]]ensus, with arginine a recognized and expected substitution. Another clone (3-4) shows a substitution at position 122** of an amino acid with a hydrophobic sulf[[p]]hydryl group (cysteine) with another having a hydrophobic, aliphatic side chain (valine). Since cysteine is most common it is included in the cons[[c]]ensus with valine noted as a potential substitution. For alignment purposes in FIG. 7.3, a gap is present at position 94 in the sequence for ALL Tm 12.84 clones, since they share the smaller, 115 residue number. Thus, as will be the case for all Tm 12.84 clones, residue position numbers in FIG. 7.3, listed after 94 will reflect this extra number assignment. Therefore, as in the example above, clone 3-4 has the valine substitution actually at position 121 from the initial methionine, as seen in ~~SEQ ID NO. 10)~~ SEQ ID NO:10).—

Please amend the paragraph beginning on page 78, line 25 as indicated.

--As more distant relatives of the gene family are considered, it is important to note the strongly conserved features of the group as these are most probably responsible for their common functions (i.e. antifreeze activity) and certainly provide clues as to their evolutionary origins. In developing the ~~concensus~~ consensus sequences, we have included the furthest members of the family (refer to FIG. 4.19 and 4.20); the ~~assessory~~ accessory gland proteins B-1 and B-2 from T. molitor, putatively thought to be pheromone binding proteins; and AFP-3(THP-12), also from T. molitor and ~~demonstated~~ demonstrated to be a small lipid carrier, but whose status as an AFP is in doubt. Additionally, note that B-1 and B-2 lack a complete open reading frame, missing both the N-terminal methionine, and a suitable, "in frame" stop codon at the C-terminus (as determined from their first translated amino acid). Nor do they have a poly adenylation signal and poly A tail. Since the comparisons are based only on partial sequences, we can expect the ~~concensus~~ consensus to change as their complete sequences are revealed. Therefore, further comparison has focused on full length members of the family.--

Please amend the paragraph beginning on page 78, line 38 as indicated.

--Every cysteine residue save the last is completely conserved in every member of the family. They are found at positions (from the initial methionine) 6, 34, 65, 105, and 122 from the initial methionine (FIG. 7.3). Regions around these cysteine residues are also conserved with particular conservation of lysine, glutamine, glutamic acid, isoleucine, and valine. When these residues are substituted in any of the family members the replacement is typically a substitution of kind, with one aliphatic amino acid replacing another, or a basic replacing a basic, and so forth. Even when the substitutions are not in kind, other aspects of the side chain chemistry are similar. For example, in AFP-3, the ~~concensus~~ consensus glutamic acid is occasionally replaced by either arginine or lysine. Although these would appear to be

opposites (basic groups for an acidic one), both groups are polar, hydrophilic, and reactive.--

Please amend the paragraph beginning on page 79, line 18 as indicated.

--Together, the conserved residues and similar substitutions form a general pattern that contributes to the special chemistry of this family of proteins, including their ability to bind to ice and prevent crystal growth. ~~SEQ ID NO. 48~~ SEQ ID NO:48 presents a full general consensus peptide sequence for the entire Tm 12.86 gene family. With this in mind, although never tested, the close similarity of the B1 and B2 *T. molitor* proteins (indeed more so than AFP-3) suggest that these will likely exhibit antifreeze activity.--

Please amend the sentence beginning on page 102, line 34 as indicated.

--For the selected *T. molitor* hemolymph samples represented in FIGS. 8.23, 8.25 and 8.30, the increase in hemolymph RI factor associated with the acclimation of *T. molitor* from summer to winter conditions is observed as leftward shifts of the regression lines.—

Please amend the sentence beginning on page 106, line 29 as indicated.

--Mathematical ~~modelling~~ modeling of recrystallization and RI.--

Please amend the paragraph beginning on page 109, line 26 as indicated.

The invention also details the use of light scattering as a means of quantifying RI as an alternative to mean largest grain size measurements, and is very amenable to automated processes (FIG. 8.3). As detailed, this method would be intended for more of a rapid screening technique with a

moderately high level of quantitation. However, in instances (including samples identified ~~throught~~ through the light scattering method) that require a high degree of quantitative accuracy, would then need evaluation via the mlgs RI dilution profile and RI factor analyses.--

Please amend the paragraph beginning on page 112, line 5 as indicated.

--Another level of automation of the RI assay is directed toward image capturing of the ice crystals viewed through the microscope for monitoring ice crystal growth over time and to report quantitative data based on what is observed in the field of view. This can be readily accomplished through either video recording using a CCD camera or through image capturing via a digital camera. Additionally, standalone image analysis software that will monitor ice crystal growth within a 256 gray-scale (or through more upscaled color monitoring) and perform size calculations on the resultant data, with particular reference to the foundation studies based on the mean largest grain size, RI dilution profiles, linear regression analyses for RI factors and ANCOVA slope analyses, will provide meaningful, reliable, biologically ~~relevent~~ relevant calibration references. Therefore at least two modes for identifying ice crystals need to be employed. The first is to monitor the largest five ice grains (possibly from 2-3 separate fields of view) over time, for assessment of composite mlgs. The second mode could monitor all the grains that are in the field of view over time. Operator-editable parameters will allow choice of measurement frequency and selection of ice grain assessment characteristics evaluated. The advantages of all this over typical generic image analysis software will be the ability to relate computer assisted images and measurement parameters to true documented foundation studies on RI behavior of a purified AFP under a variety of different assaying conditions (as detailed in this invention). Moreover, it can facilitate implementation and testing of the mathematical modeling equations described and thereby also allow for a systems level approach and predictive

theories to recrystallization behavior of solutions and the impact of ice growth suppressing peptides.--

Please amend the paragraph beginning on page 112, line 31 as indicated.

--With a combination of upscaling for multiple samples and image capturing and analysis of their ice crystal grain sizes, this invention is likely to have numerous industrial and commercial uses for detecting and quantifying ice recrystallization, and also provide the impetus for reducing or eliminating ~~deleterous~~ deleterious ice with addition of AFPs. To name just a few examples, it is envisioned that the frozen food industry and ice cream manufacturers could better monitor and improve shelf life of their products, and the meat and poultry industries which also requires extended storage of partially thawed meats and poultry would be particularly suited for such implementation. Additionally, similar monitoring would provide important improvements for gauging the longevity of tissue cryopreservation and extended storage of synthetically engineered tissues, while predictive rates and therefore, selected control of localized ice crystallization would improve implementation in cryosurgury. Moreover, this would enable more large scale screening of the effectiveness of current and newly designed de-icing solutions, including those containing natural or recombinant and/ other organically synthesized AFPs.--

Please amend the paragraph beginning on page 118, line 3 as indicated.

--An advantage of the microcapillary method is that if offers great consistency of thermal hysteresis measurements due to controlled measurement parameters, and can detect thermal hysteresis activity as small as 0.02 C, corresponding to the estimated resolution of the method. However, this method is subject to seed crystal size variation, which can influence thermal hysteresis ~~measurment~~ measurement, and requires experimenter skill to minimize this effect.--

Please amend the paragraph beginning on page 121, line 19 as indicated.

--In vitro translation. Isolated MRNA was subjected to in vitro translation using an in vitro translation kit (Stratagene) and following the procedure provided by manufacturer. In general, 2 .mu.l (1 .mu.g/.mu.l) mRNA isolated from T. molitor was incubated at 68.degree. C. for 30 seconds, then 2 .mu.l .sup.35S-methionine-1200 Ci/mmole (DuPont NEN) was immediately added. DEPC water (1 .mu.l) was added to the final volume of 5 .mu.l. Then 20 .mu.l of thawed and well-mixed lysate of rabbit reticulocyte was added to the reaction, which was mixed thoroughly and incubated in a 30.degree. C. heat block for 1 hr. The translation products were precipitated by TCA precipitation assay. The pellet was resuspended in electrophoresis buffer and loaded onto a SDS-PAGE gel for electrophoresis and autoradiography. If it was necessary to store the in vitro translation products, ~~electrophrocsis~~ electrophoresis buffer was added and the samples were boiled for 5 min, then frozen at -80.degree. C.--

Please amend the paragraph beginning on page 122, line 26 as indicated.

--Electrophoresis analysis on SDS-PAGE gel and Fluorography. Translation products and immunoprecipitation products were ~~analysized~~ analyzed by electrophoresis on 0.8 mM of SDS-PAGE polyacrylamide gel following the protocol detailed in Example 1, Section 3 using either a 15%; 17% or 20% resolving gel in conjunction with a 5% stacking gel. The gel was fixed and stained in the 10% methanol, 10% glacial acetic acid solution with 0.1 .mu./ml Coomassie brilliant blue (R-250) for one hour and then destained in the 10% glacial acid and 50% methanol solution. The destain solution was changed after 5, 10 and 60 minutes. After destaining was complete, the gel was transferred into the enhance solution (EN.sub.3HANCE.TM., Biotechnology System, NEN Research Product) for one hour and then washed with distilled water. Finally, the gel was placed onto a piece of filter paper and dried under heat (60-70.degree. C.) and vacuumed on a slab gel

drying apparatus. The dried gel was exposed to Kodak X-ray film (Biomax, MR or X-omat RP) overnight or longer depending on the count of the radioactivity from TCA incorporation result. The film was developed according to the instructions provided.--

Please amend the paragraph beginning on page 126, line 30 as indicated.

--Preparation of protein samples from positive clone. To test whether the ~~recombinent~~ recombinant protein expressed from the positive clones had antifreeze activity, protein was extracted from clones grown in 100 ml of LB containing kanamycin (50 .mu.g/ml) with agitation (250 rpm) at 37.degree. C. When OD600 reached about 0.2-0.5, IPTG (1-2 .mu.M/ml) was added to the culture to induce the expression of ~~recombinent~~ recombinant protein. The culture was incubated for additional 5 hours and then pelleted in 1500 g for 10 min. The pellet (about 1 gram) was resuspended in 5 ml protein extraction buffer (50 mM Tris, pH 8.0, 1 mM of EDTA, 100 mM NACl). Then, 4 .mu.l of 0.1 M PMSF (phenylmethylsulfonylfluoride), and 80 .mu.l of lysozyme (10 mg/ml) was added and the sample was stirred 20 minutes at room temperature. 4 mg of deoxycholate was added and incubated at 37.degree. C. until the solution became very viscous (approximately for 15 minutes). Then 20 .mu.l of DNase I (1 mg/ml) was added and stirred at room temperature for about 30 minutes (until the solution was no longer viscous). The solution was centrifuged for 15 minutes at 10K rpm. The pellet was washed with the extraction buffer plus 0.5% Triton and 10 mM EDTA, and then incubated for 10 minutes at room temperature, and centrifuged for 15 minutes at 10 K rpm. The pellet was dissolved in teflon homogenizer containing 2.5 ml solubilization buffer (8 M urea deionized, 50 mM tris, pH 8.0, 0.01% Triton, 200 mM NaCl) and incubated for 1.5 hours at room temperature with shaking. The solution was then centrifuged for 15 minutes at 10K rpm, and supernatant was diluted to approximately 500 .mu.g/ml protein with renaturation buffer (6 M urea deionized, 50 mM Tris, pH 8.0, 0.01% Triton, 0.20 M NaCl, 1 mg reduced glutathione, and

0.05 mM oxidized glutathione) and stirred for 1.0 hour at 4.degree. C. The renatured sample was then changed for 12 hours, and then 6 hours against each 300 ml of 50 mM tris at pH 8.0, 0.01% Tween 80, 200 mM NaCl, 1 mM of reduced glutathione, and 0.05 mM of oxidized glutathione. Then in order to get rid of the salt the solution was further dialyzed against dH2O with changing water every six hours for three times. Finally, the solution was lyophilized and resuspended in a small amount of dH2O (about 20 .mu.l).--

Please amend the paragraph beginning on page 132, line 6 as indicated.

--The reaction mixtures were then subjected to thermal cycling on an MJ Research PTC-200 Peltier Thermal Cycler, creating dye-terminated complementary DNA extension strands. The thermal cycler first heat samples to 96.degree. C. for 30 seconds (denatures dsDNA into single strands), followed by cooling to 50.degree. C. for 15 seconds (allows primers to bind ssDNA), then heating to 60.degree. C. for 4 minutes (primer extension: polymerization of ~~complentary~~ complementary DNA strands). These three steps are repeated in sequence 25 times. After thermal cycling, the newly synthesized DNA extension strands were purified using Centri-sep spin columns (Princeton Separations) which function as gel filtration columns to remove unused nucleotides from the reaction mixtures. Briefly, the spin columns were prepared according to the manufacturer's recommendations by hydrating the gel beads in 0.8 ml H.sub.2O for 30 minutes, then allowing the liquid to drain from the column by gravity. Liquid remaining in the column was drained by centrifuging the column at 750 g (3000 rpm using the Eppendorf Model 5415C) for two minutes. The 20 .mu.l reaction mixture volume was pipetted onto the top of the gel matrix, followed by placement of the column into a collection tube and centrifugation at 750 g for 2 minutes. The resultant liquid expelled into the collection tube (containing purified DNA strands) was saved and then dried using a Savant Speed-vac for 20 to 30 minutes. Care was taken not to excessively dry the DNA, since this might interfere with subsequent rehydration steps. The collection tube with DNA was

then wrapped in aluminum foil (to avoid exposing the nucleotide-conjugated dyes to light) and stored at -20.degree. C. in preparation for analysis using the ABI Genetic Analyzer.—

Please amend the paragraph beginning on page 145, line 6 as indicated.

--Part D: Sequence ~~Comparision~~ Comparison to Examine Relationships within the Tm12.86 Multigene Family--

Please replace the paragraph beginning on page 145, line 8 with the following amended paragraph. Note: The words Sequence Data and T. molitor were underlined in the original specification. Added material starts with the word Genbank's.

--Sequence Data. DNA sequence data from *T. molitor* was obtained from cDNA clones selected from a *T. molitor* cold acclimated cDNA library with an antibody to the T. molitor AFP Tm 12.86. Several positive clones were sequenced using the ABI Prism model 310 DNA sequencer. The clones concentrated on are Tm 13.17 (Example 2), 2-2 and 2-3 (Example 3), and 3-4, 3-9, and 7-5 (Example 4, Part C). Also available were the N-terminal amino acid sequence of Tm 12.86 (Example 1), and the nucleotide sequence and predicted amino acid sequence of AFP-3, B1 and B2, and other sequence data obtained from GenBank's database located on the National Center for Biotechnology Information's website - www.ncbi.nlm.nih.gov [~~GenBank (www.ncbi.nlm.nih.gov)~~]. --

Please amend the paragraph beginning on page 158, line 11 as indicated.

--Derivation of ~~Conceensus~~ Consensus Sequences for the Tm 12.86 Gene and Protein Families--

Please amend the paragraph beginning on page 158, line 12 as indicated.

--In developing cons[[c]]ensus sequences for the genes and proteins of the Tm 12.86 family (cladistic tree shown in FIG. 4.20), careful attention was made to the types of substitutions and the chemistry involved. Both a full generic cons[[c]]ensus sequence was identified for the entire Tm 12.86 gene family encoded proteins, and consensus sequences for the nested genes within the family are also described (i.e. cons[[c]]ensus sequence for Tm 12.84-6 like, consensus sequence expanded to include Tm 13.17 like, cons[[c]]ensus sequence expanded to include B1/B2 like, and cons[[c]]ensus expanded to include AFP3 like, genes and their encoded proteins ~~(SEQ ID NO. 44-48).~~ (SEQ ID NOS:44-48).—

Please amend the paragraph beginning on page 158, line 20 as indicated.

--The following letter designations used in deriving these ~~concensus~~ consensus sequences are as specified below. In the ~~concensus~~ consensus gene sequences, (FIG. 7.2) there are the letters for the four bases, A, G, C, and T. In addition, N is used to designate "any nucleotide", Y is used to designate "any pyrimadine" (C or T), and R is used to designate "any purine" (A or G). In the ~~concensus~~ consensus, we have included the designations A/T, T/A, G/C, and C/G. These reflect the special relation these pairs of bases have in the anitparallel strands of DNA. In fact, if a T is substituted for A, for example, the opposite strand then must be substituted A for T. The base pair at that position is retained, though the precise sequence has changed and may affect the protein when translated. Conversely, if C is substituted for T (pyrimadine substitution), then the opposite strand must substitute G for A (purine substitution). The sequence is changed and the original pair is completely ~~elimintated~~ eliminated. The chemistry of the strands also changes since the G-C bond is stronger than the A-T bond. The ~~concensus~~ consensus sequences listed for each grouping (among Tm 12.84; expanded to include Tm 13.17 like, then B1 like, and then AFP-3 like) list the most representative ~~concensus~~ consensus sequence and positions and types of substitutions occurring or deemed acceptable

(FIG. 7.2).--

Please amend the paragraph beginning on page 158, line 35 as indicated.

--With protein sequences, convention assigns a three letter abbreviation or a single letter to each amino acid. The three letter abbreviation is more tractable to describing substitutions and fits nicely with each three letter codon in the gene sequence, yet it's bulk in generating column groupings was undesirable, so single letter assignments for each amino acid was chosen for generating the protein ~~concensus~~ consensus for the Tm 12.86 family (FIG. 7.3). However, no convention has been developed for describing substitutions in the single letter system. We have chosen to designate substitutions as to chemical class and hydrophobicity classifications. Refer to FIG. 7.1 for one and three letter designations of amino acids, and their chemical class and other key characteristics. The ~~concensus~~ consensus protein sequences listed for each grouping (among Tm 12.84; expanded to include Tm 13.17 like, then to B1 like, then AFP-3 like, and then an identified general ~~concensus~~ consensus peptide) lists the most representative ~~concensus~~ consensus sequence to encompass that grouping and positions and types of substitutions occurring or deemed acceptable.—

Please amend the paragraph beginning on page 160, line 15 as indicated.

--A coverglass was placed at the bottom of the cold stage to form a holding chamber; ice samples were positioned on a small polypropylene ring (cut from the top of a 0.5 ml microfuge tube) at the bottom of the chamber, which was then sealed with a second coverglass. The temperature within the chamber was monitored using a Type T thermocouple needle microprobe (Physitemp type MT-26/2) with digital thermometer (Physitemp BAT-10RLOP) and a second Type T thermocouple sensor (Physitemp type MT-4) immersed in an ice-water bath for differential ~~temperture~~ temperature measurements.--

Please amend the paragraph beginning on page 165, line 12 as indicated.

--The written Sequence Listings for ~~SEQ ID NO's 1-48~~ SEQ ID NOS:1-48 (pages 166-221) are attached herein with the Submission of the Computer Readable Copy.--